

Article

Physiological and biochemical markers of the gender-specific sensitivity to epileptogenic factors, delayed consequences of seizures and their response to vitamins B1 and B6 in a rat model of epilepsy

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Abstract

Disturbed metabolism of vitamins B1 or B6, which are essential for neurotransmitters homeostasis, may cause epilepsy. Our study aims at revealing therapeutic potential of vitamins B1 and B6 in epilepsy by estimating effects of their combined administration on a seizure and its consequences in rats subjected to pentylenetetrazole (PTZ). The PTZ dose dependence of a seizure and its parameters according to Racine's scale along with delayed physiological and biochemical consequences next day after the seizure are assessed regarding sexual dimorphism in epilepsy. PTZ sensitivity is stronger in the female than male rats. Next day after a seizure, gender differences in behavior and brain biochemistry arise. The induced gender differences in anxiety, exploratory and locomotor activity correspond to disappearance of gender differences in the brain GABA, aspartate, alanine and serine, with appearance of those in glutamate, glutamine and tyrosine. PTZ decreases the brain malate dehydrogenase activity, glutamine and urea in the males, and phenylalanine in the females. Administration of vitamins B1 and B6 24 h before PTZ delays a seizure in female rats only. This desensitization is not observed at short intervals (0.5-2 h) between the vitamins and PTZ administration. With the increasing interval, the pyridoxal kinase (PLK) activity in the female brain decreases, suggesting that the PLK downregulation by vitamins contributes to the desensitization. Delayed effects of vitamins and/or PTZ are mostly gender-specific and interacting. Our findings on the gender differences in sensitivity to epileptogenic factors, action of vitamins B1/B6 and associated biochemical events have medical implications.

Keywords: amino acid neurotransmitter, glutamate, GABA, pentylenetetrazole, vitamin B1, vitamin B6.

Introduction.

Epilepsy is a common and predominantly polygenic disease characterized by occurrence of repeated seizures triggered by abnormal brain activity, generally due to a disbalance between neuronal excitation and inhibition. In more than a half of the cases, the cause of epilepsy remains unknown. Some epilepsies, called vitamin-dependent, belong to rare genetic disorders, caused by mutations in the genes involved in the metabolism of vitamins B1 and B6 [1-5]. Normalization of the organismal vitamin status in these types of epilepsy has a therapeutic effect, eliminating seizures that cannot be treated with known antiepileptic drugs. Vitamins B1 and B6 may also contribute to other, non-monogenic types of epilepsy. Although the role of the vitamins in the pathology remains poorly understood, a number of enzymes participating in the metabolism of glutamate and GABA, are altered in epilepsy. Those include not only known targets of vitamin B6 (transaminases and decarboxylases), but also the enzymes (e.g. glutamate dehydrogenase (GDH), malate dehydrogenase (MDH), glutamine synthetase (GS) and others) whose function may be regulated by vitamin B1 beyond the coenzyme action of this vitamin on the thiamine-diphosphate-dependent enzymes, such as dehydrogenases of 2-oxo acids [6-12]. Subclinical hypovitaminosis of B1 or B6 may arise in patients treated with widely used medications, including antiepileptic drugs [13-16], potentially facilitating the development of non-monogenic epilepsies. Hence, upon dysfunction in the vitamin metabolism, either acquired or caused by genetic defects, administration of vitamins B1 and/or B6 may mitigate epileptic seizures and their long-standing consequences. This work aims to characterize potential therapeutic effect of the administration of these vitamins in a rat model of epilepsy, as well as effects of a seizure on the brain central metabolism dependent on vitamins B1 and B6.

Our study takes into account physiological observations on sexual dimorphism in epilepsy [17]. To induce an epileptic seizure, we employ a widely used model of pentylenetetrazole (PTZ) administration. Binding to GABA receptors, PTZ causes disbalance between excitatory and inhibitory pathways of neurotransmission [18,19]. Physiological manifestations of epilepsy are analyzed not only characterizing severity of a seizure, but also assessing post-epileptic behavioral and ECG parameters. To study potential therapeutic effects of high doses of vitamins in epilepsy, administration of vitamins B1 and B6 is combined, in view of the B1-dependent regulation of B6 metabolism in animals [11]. The administered doses of the vitamins are equivalent to those known from medical applications in human patients. To characterize molecular mechanisms and pathways involved, the physiological assessment is combined with the analysis of the consequences of a seizure for the brain biochemistry. In this part, the vitamin-dependent enzymes crucial for the brain metabolism of glutamate and GABA [1,2,20-24], are assayed, and the brain amino acid profiles [21,22] quantified. As a result, we reveal gender differences in sensitivity to PTZ administration,

biochemical markers of such differences, a dual gender-specific effect of the administration of vitamins B1 and B6 on a seizure, and association of the protective effect of the vitamins on a seizure with the regulation of the brain PLK activity.

Results

Gender differences in the susceptibility of animals to the epilepsy inducer PTZ and to the delayed consequences of an epileptic seizure

The scheme of administration of pentylenetetrazole (PTZ) has been selected to minimize mortality, simultaneously achieving a strong manifestation of the clonic and tonic seizures according to the Racine scale (see Materials and Methods). Accordingly, the PTZ dose received by individual animals varies depending on their individual susceptibility (Supplementary Figure S1). Sensitivity of the male and female rats to the epilepsy inducer is characterized by an average PTZ dose and pathophysiological parameters of the seizure development, shown in Figure 1 A. The average PTZ dose is practically the same for the female and male rats: 58 ± 4 vs 60 ± 4 mg/kg, respectively (Fig. 1 A). However, the mean seizure score is significantly ($p = 0.05$) lower in the male rats, compared to the female ones (Fig. 1 A). In addition, male rats experience convulsive twitching ($p = 0.05$) and tonic convulsions ($p = 0.08$) app. 4 min later, compared to the female rats (Fig. 1 A). Thus, the obtained results indicate that female rats are more sensitive to the epileptogenic action of PTZ than male rats.

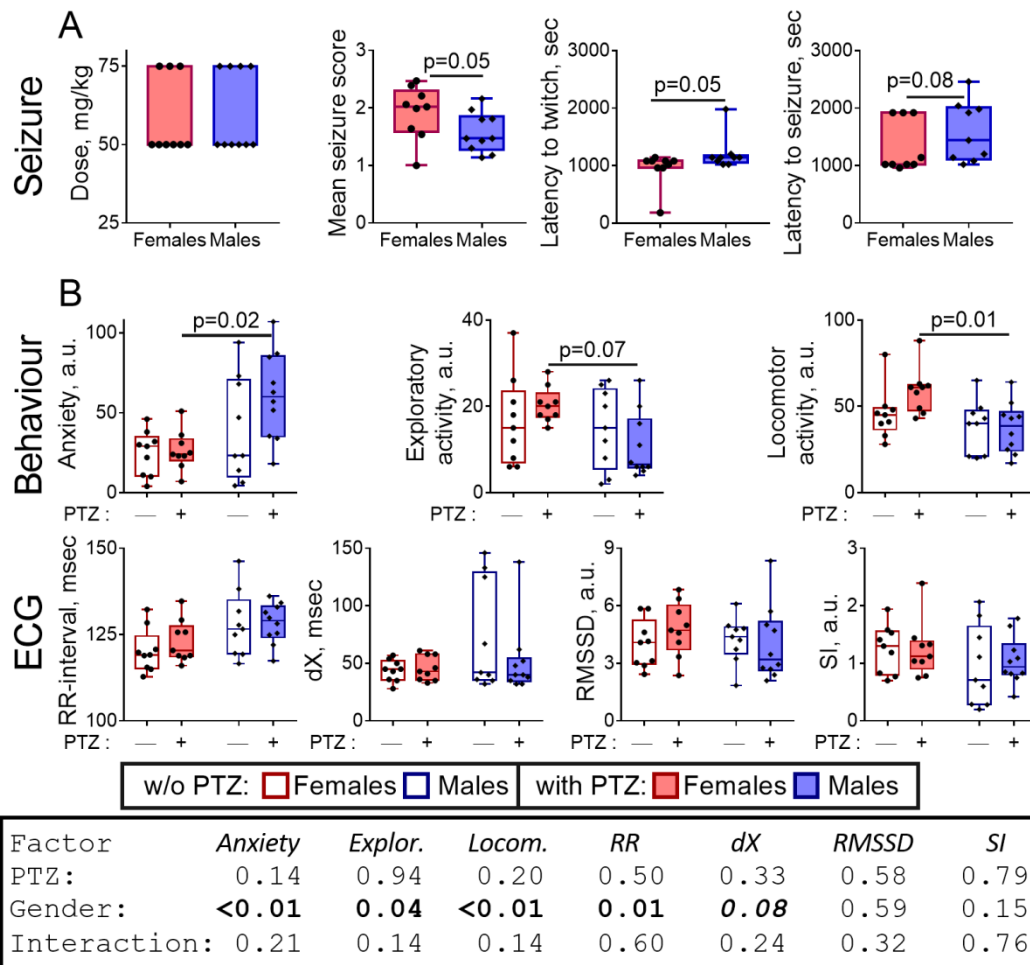


Figure 1. Gender-dependent parameters of the PTZ-induced seizure (A) and its delayed physiological effects (B), compared to the control animals. The assayed parameters are indicated on the Y axes. (A) – “Dose” refers to the total PTZ dose received by an animal for the seizure induction; “Mean seizure score” is determined during the 45 minutes of the observation; “Latency to twitch” defines the time to the first myoclonic twitch (3 points by Racine’s scale, see “Materials and methods”); “Latency to seizure” defines the time to the first tonic seizure (4 points by Racine’s scale, see “Materials and methods”). (B) – Behavioral and ECG parameters of the rats next day after a seizure, compared to the control groups. The parameters of anxiety, exploratory activity and locomotor activity are obtained from the “open field” test as described in “Materials and methods”. The ECG parameters include the length of the RR interval, the heart variability rate dX, the RMSSD and stress (SI) indexes, estimated as described in “Materials and methods”. P-values are determined using the Mann-Whitney test (A) and ANOVA (B) (see “Materials and methods”).

Next day after a seizure, gender differences in anxiety ($p = 0.02$), exploratory activity ($p = 0.07$) and locomotion ($p = 0.01$) arise, that are absent in the control groups (Fig. 1 B). In contrast to the behavioral parameters, ECG does not reveal significant changes in the rats after the seizure (Fig. 1 B). Obviously, significant gender differences in anxiety, exploratory activity and locomotion after a seizure represent a cumulative effect of minor and statistically insignificant shifts exhibited

by each of the sexes in response to PTZ. This view is confirmed by two-way ANOVA pointing to the statistical significance of the factor of sex in the PTZ-induced changes.

Analysis of biochemical markers in the cerebral cortex of rats next day after a seizure did not reveal a significant effect of PTZ on the activity of tested enzymes of the brain neurotransmitter metabolism, with the exception of a decrease in the MDH activity ($p=0.03$) in males (Fig. 2). At the same time, significant ($p = 0.01$) interaction of PTZ and gender in the analysis of MDH activity by two-way ANOVA corresponds to the opposite directions of PTZ effects on MDH activity in the male and female rats (Fig. 2). The two-way ANOVA also reveals trends ($p = 0.06$) to a slight increase in PDHC activity by PTZ in both sexes, and to a lower GDH activity in the male rats, compared to the female rats, independent of PTZ action (Fig. 2).

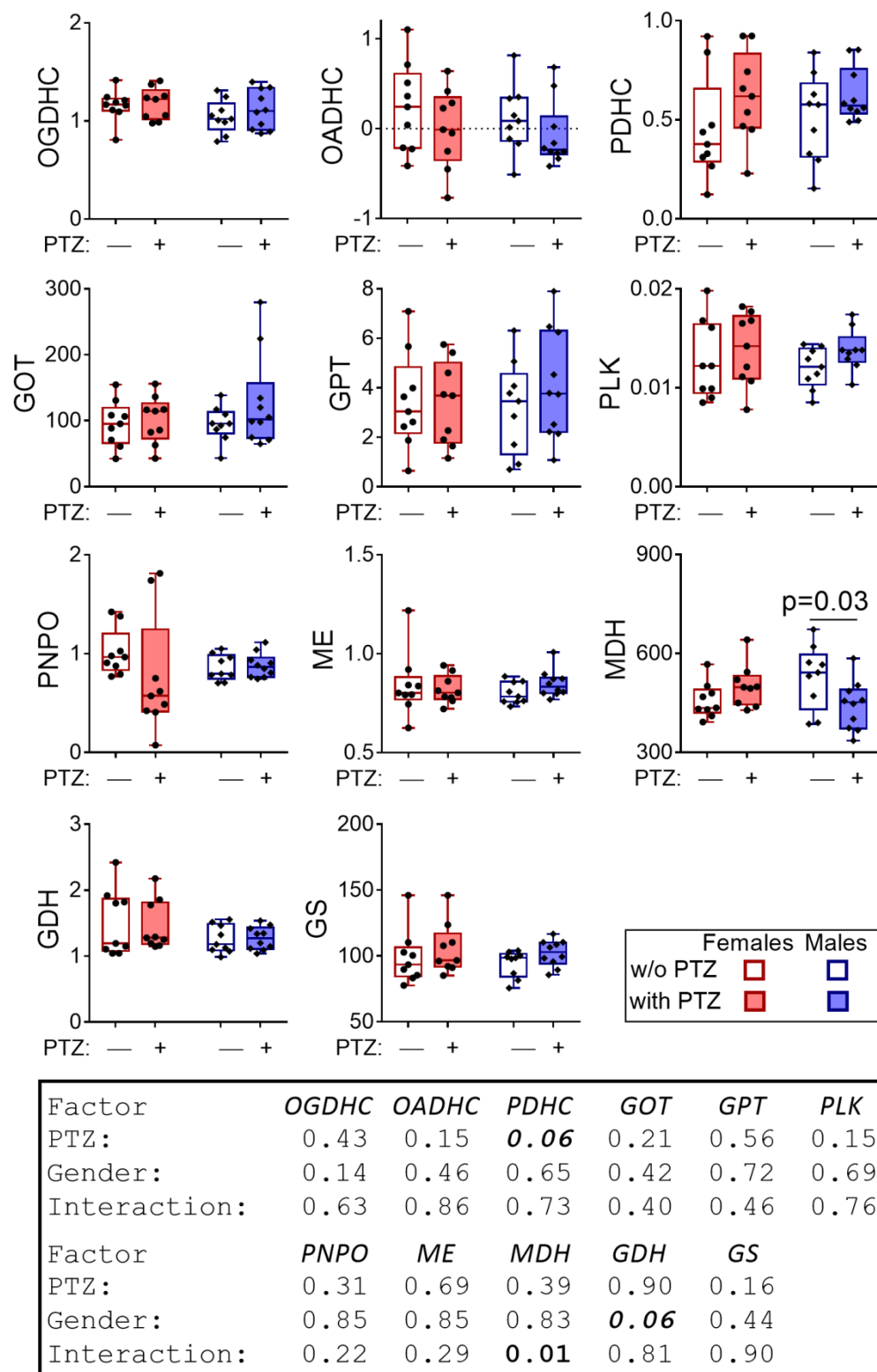


Figure 2. Delayed effect of the PTZ-induced seizure on the activities of cerebral cortex enzymes in the male and female rats, compared to the control groups. The activities are presented as micromole/min per g of tissue, with the exception for PNPO, whose activity is in nanomole/min per g of tissue.

Noteworthy, the enzymatic reaction rates *in vivo* depend on the local concentrations of metabolites. These concentrations are determined by the state of the metabolic network and usually are far from the saturating concentrations employed for enzymatic assays *in vitro*. Therefore, the minor gender-dependent differences in the GDH activity and the reactivity of MDH to PTZ, which are determined *in vitro* upon saturation with substrates and coenzymes (Fig. 2), may be amplified upon quantification of the levels of metabolites dependent on these enzymatic activities *in vivo*. Indeed, analysis of the levels of free amino acids in the brain reveal multiple gender-dependent PTZ effects on the metabolites. As seen from Figure 3, PTZ eliminates pre-existing gender differences in the levels of aspartate, alanine, serine and GABA, inducing those in the levels of glutamate, glutamine and tyrosine (the amino acids are listed in decreasing order of significance of the gender differences). The gender-specific effects of PTZ, such as decreases in glutamine and urea in the brain of male rats, and phenylalanine in the brain of female rats, are less abundant. Accordingly, the two-way ANOVA indicates that the gender factor is essential for 12 out of 15 estimated metabolites, while PTZ is essential for only 5 out of 15 ones, with the levels of glutamate and urea revealing trends for an interaction between the two factors (Fig. 3).

In summary, both the gender differences in the levels of free amino acids in the rat brain and the amino acid levels themselves respond to the action of PTZ. The gender-specific responses of the brain biochemical parameters (Figs. 2, 3) correspond to the different sensitivity of the sexes to PTZ (Fig. 1 A) and the post-seizure induction of behavioral differences between the sexes (Fig. 1 B). In particular, the PTZ-induced changes in the sexually dimorphic pattern of the brain amino acids (Fig. 3) correspond well to the PTZ-induced gender differences in behavior (Fig. 1 B).

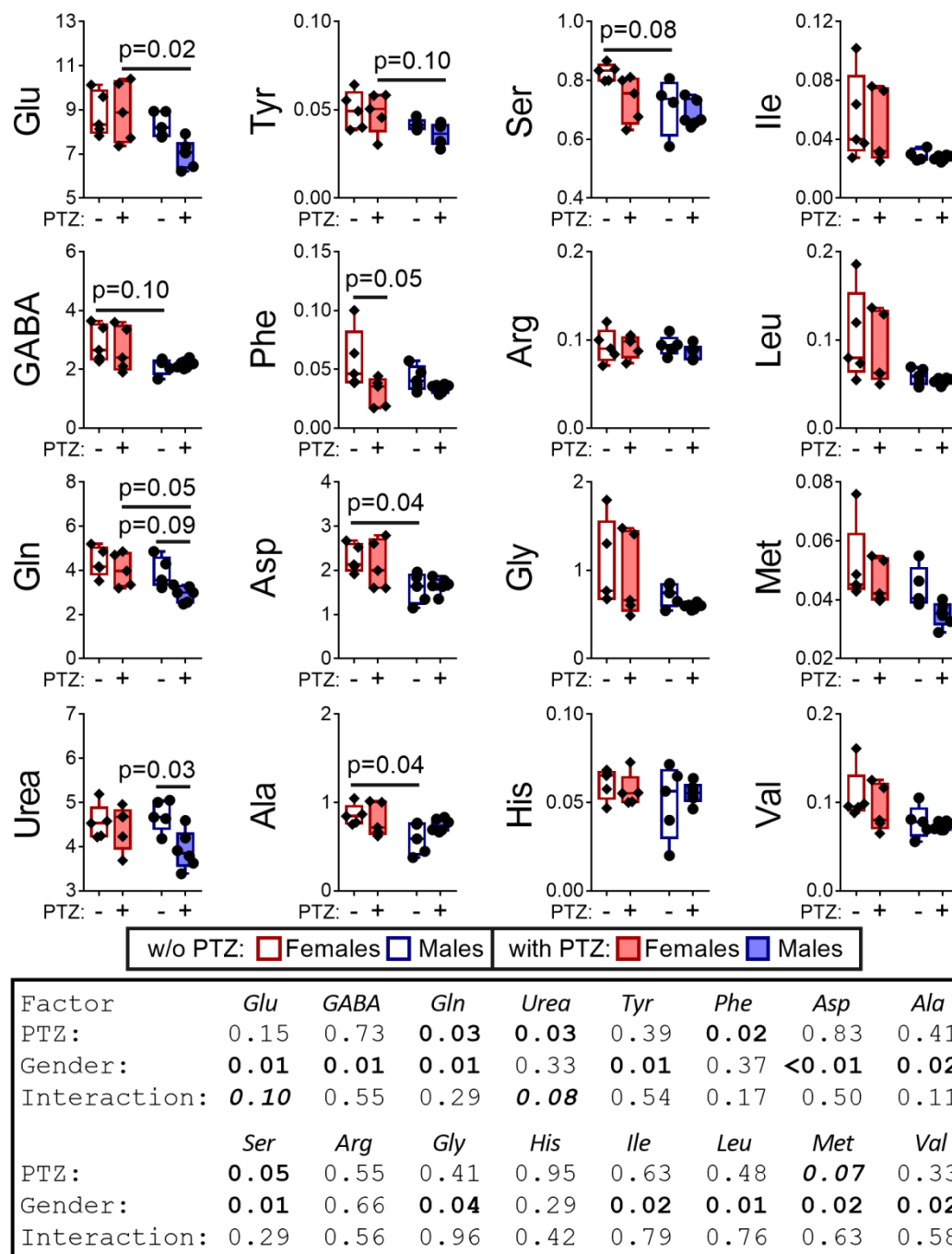


Figure 3. Delayed effect of the PTZ-induced seizures on the levels of amino acids and urea in the cerebral cortex of the male and female rats, compared to the control groups. The content of free amino acids and urea is in micromoles per g of tissue.

Gender effects of combined administration of vitamins B1 and B6 on the severity of a seizure and its delayed consequences.

In females, the combined administration of vitamins B1 and B6 24 hours before PTZ increases the time until the twitching ($p = 0.04$) or seizure onset ($p = 0.06$), but in males these parameters remain unaffected (Fig. 4 A). Thus, the higher the animal susceptibility to the epileptogenic action of PTZ, the greater the animal sensitivity to the protective action of vitamins against such action. Besides,

the vitamins administration tends to increase gender differences in seizure score ($p = 0.1$), simultaneously abrogating a tendency to gender difference in the twitching onset time (Fig. 4 A).

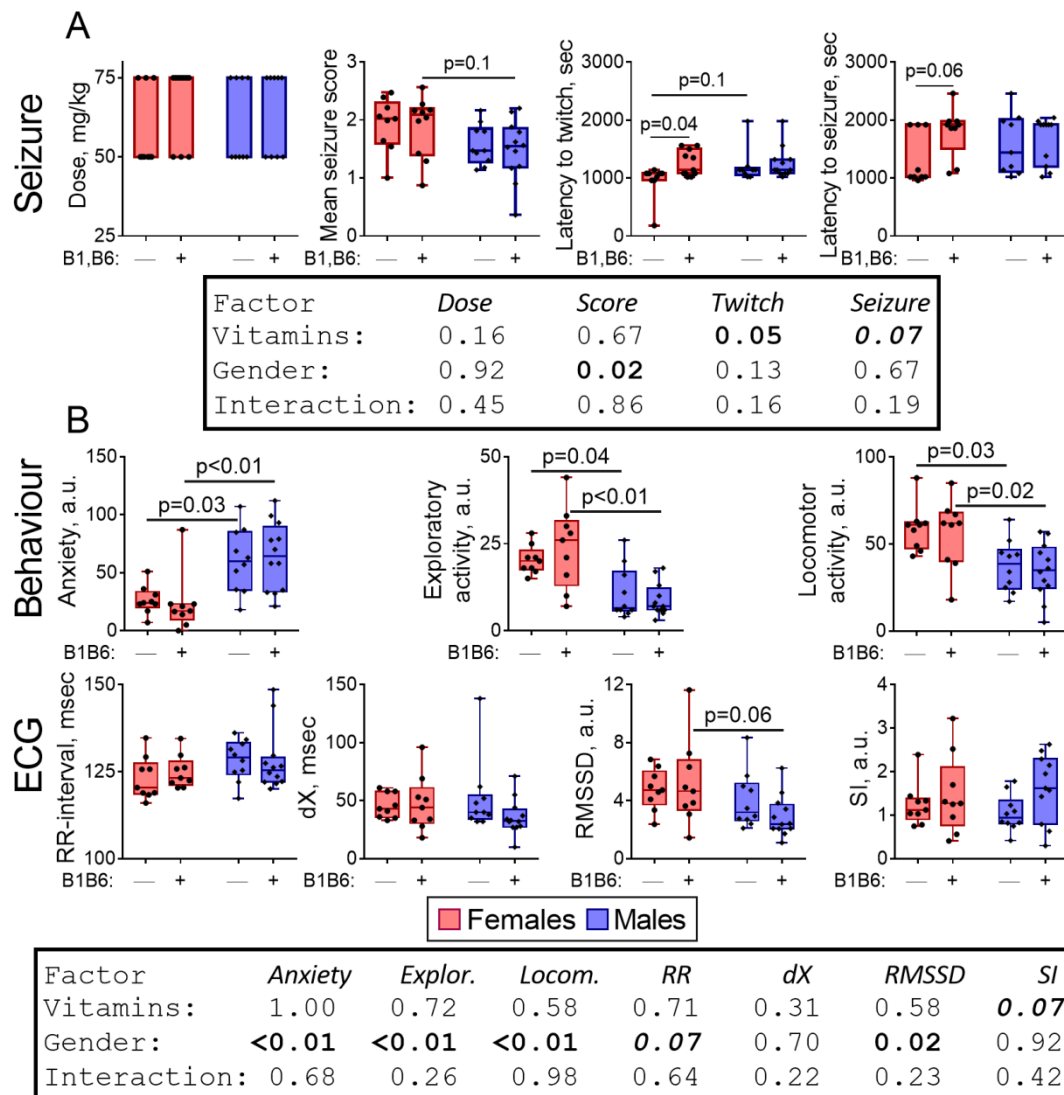
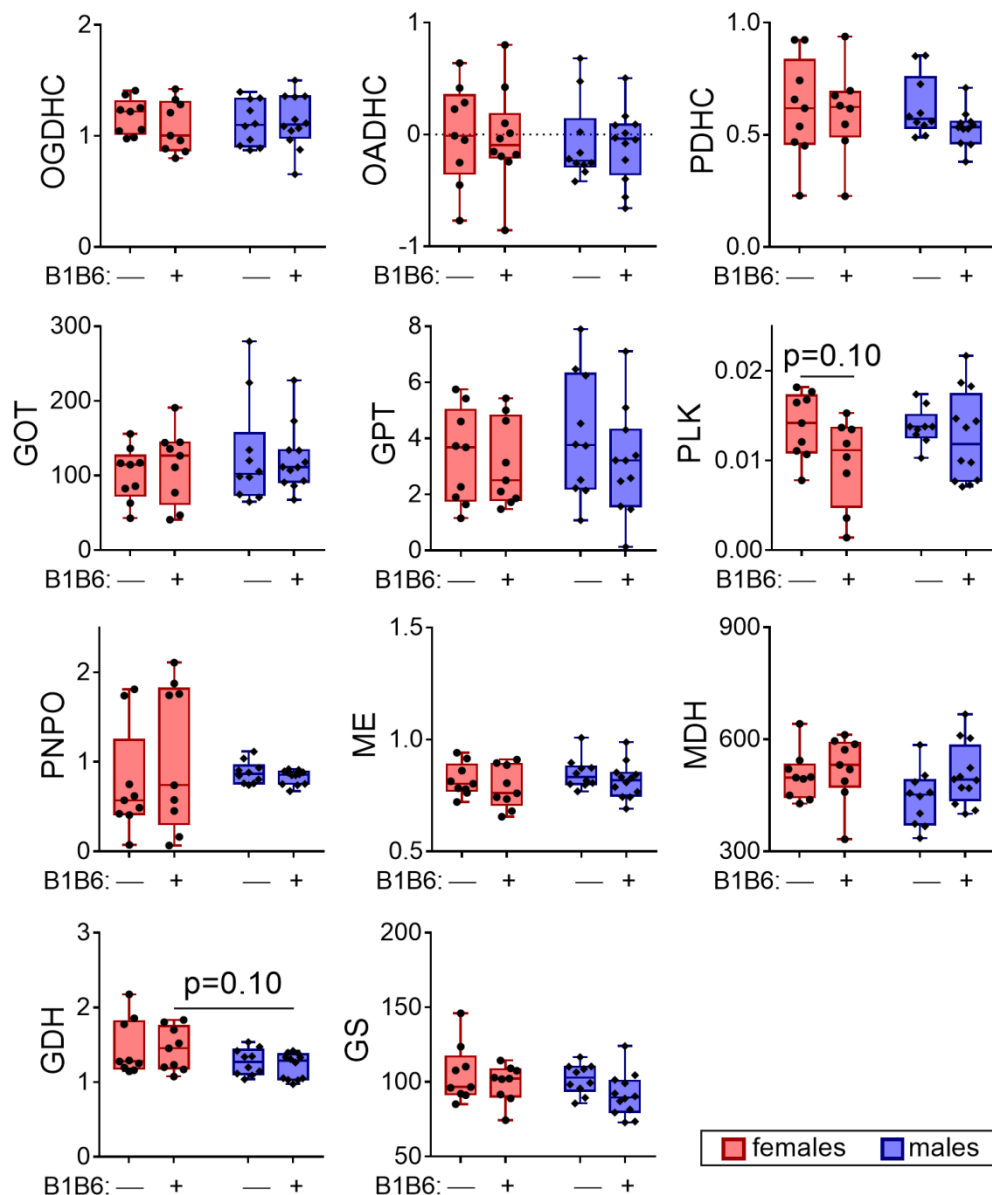


Figure 4. Gender-dependent effects of the administration of vitamins B1 and B6 on the parameters of the PTZ-induced seizure (A) and its delayed physiological effects (B). The assayed parameters are indicated on the Y axes and described in more details in Figure 1 and “Materials and methods”.

As for the delayed effect of the PTZ-induced seizure on behavior and ECG parameters, vitamins slightly enhance the gender differences arising after the seizure (Fig. 4 B). This is observed from an increase in significance of the behavioral differences between the male and female rats after the administration of both PTZ and vitamins ($p \leq 0.02$), compared to similar groups with no administered vitamins ($p \geq 0.03$) (Fig. 4 B). Besides, a tendency ($p = 0.06$) towards gender differences in the RMSSD parameter of ECG emerges after the administration of both vitamins and PTZ, which is not observed under the action of PTZ alone (Fig. 4 B).



Factor	<i>OGDHC</i>	<i>OADHC</i>	<i>PDHC</i>	<i>GOT</i>	<i>GPT</i>	<i>PLK</i>
Vitamins:	0.58	0.81	0.32	0.87	0.33	0.06
Gender:	0.92	0.57	0.58	0.31	0.53	0.33
Interaction:	0.45	0.95	0.48	0.60	0.58	0.30
Factor	<i>PNPO</i>	<i>ME</i>	<i>MDH</i>	<i>GDH</i>	<i>GS</i>	
Vitamins:	0.44	0.19	0.10	0.67	0.07	
Gender:	0.72	0.24	0.13	0.02	0.22	
Interaction:	0.28	0.97	0.39	0.81	0.62	

Figure 5. Effects of administration of vitamins B1 and B6 on the activities of cerebral cortex enzymes in the male and female rats next day after the PTZ-induced seizure. The activities of enzymes are expressed in micromole/min per g of tissue, with the exception for the PNPO activity, expressed in nanomole/min per g of tissue.

Biochemical analysis of the rat brain also shows more pronounced changes upon the combined action of PTZ and vitamins in comparison with the action of PTZ alone. Specifically, comparison

of the enzymatic activities in the PTZ-treated groups with or without the vitamins administration, reveals that the vitamins increase a gender difference in the brain GDH activity and decrease the PLK activity in females (Fig. 5). According to two-way ANOVA, regardless of gender, the vitamins are essential for the induction of changes in the GS and MDH activities (Fig. 5).

Similar to the effects of PTZ alone, upon the combined administration of the vitamins and PTZ, the brain metabolite levels undergo much more pronounced changes than the brain enzymatic activities do. When comparing the effects of vitamins in the PTZ-treated animals (Fig. 6) to the action of PTZ on the control animals (Fig. 3), partial reversal of the PTZ effects by vitamins is remarkable. The PTZ-induced or abrogated gender differences in the levels of cerebral glutamate, GABA, glutamine and alanine (Fig. 3) are returned to the control pattern in the vitamin-supplemented animals exposed to PTZ (Fig. 6). This is accompanied by increases in the levels of glutamate and urea in the vitamin-supplemented PTZ males vs those without vitamins (Fig. 6), the effects being opposite to those of PTZ (Fig. 3). However, the PTZ-perturbed gender relationships in the levels of tyrosine, aspartate and serine (Fig. 3) are not normalized by the vitamins administration (Fig. 6). In the female brain, the vitamins do not return the phenylalanine level (Fig. 6) to its control value which is decreased by PTZ (Fig. 3) either. Moreover, the combined administration of vitamins and PTZ induces additional gender differences in the levels of histidine, glycine, methionine and the branched-chain amino acids (Fig. 6), which are not statistically significant in either the control or PTZ-treated rats (Fig. 3). Thus, administration of vitamins B1 and B6 24 h before PTZ restores gender relationships in some of the amino acid levels, simultaneously enhancing the gender differences in other amino acid levels. While the former effect of the vitamins administration to the PTZ-treated animals is observed in the biochemical parameters only, the vitamins-induced enhancement of gender differences in the brain levels of histidine, glycine, methionine and branched-chain amino acids corresponds well to the enhancement of gender differences in physiological parameters (Fig. 4 B).

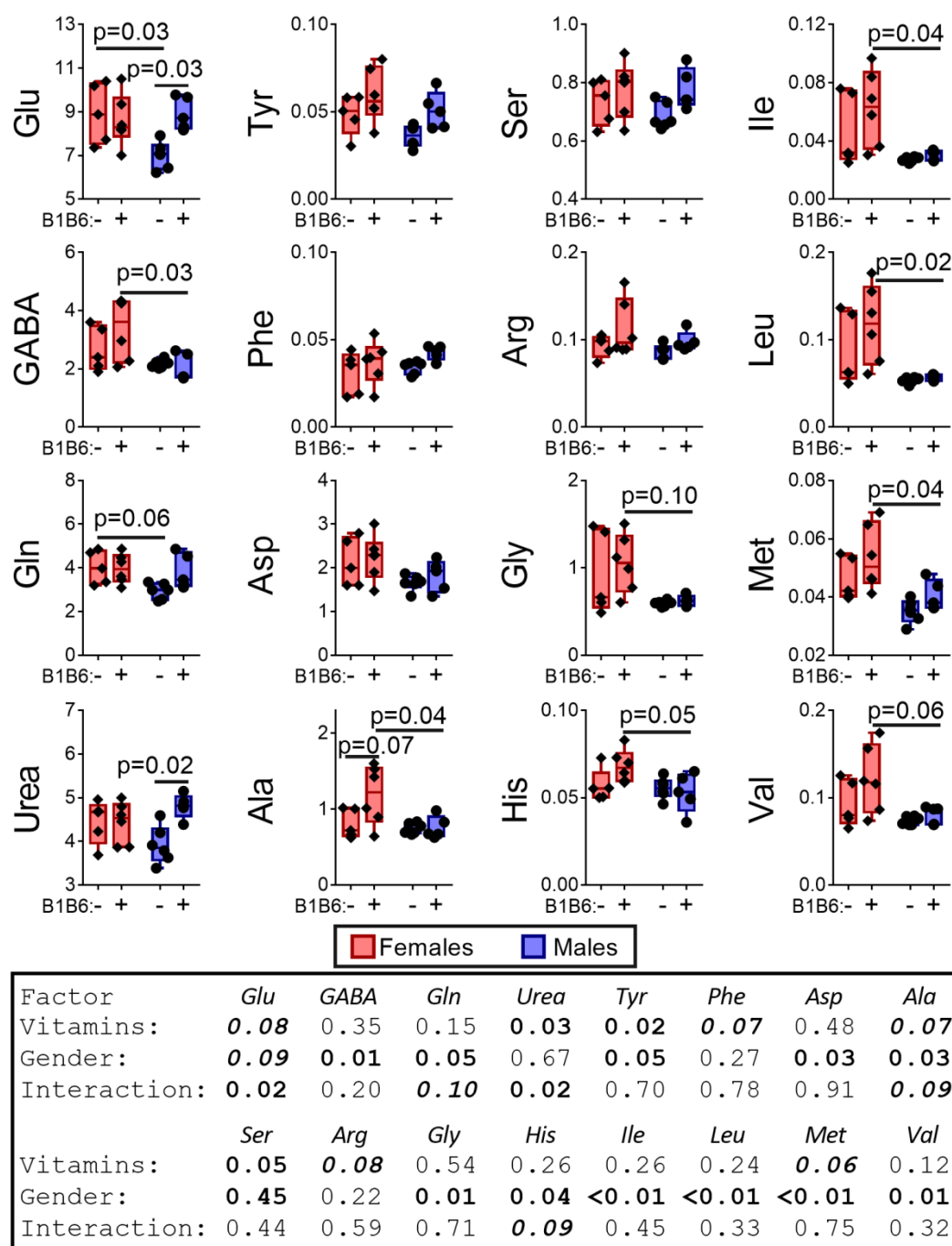


Figure 6. Effects of administration of vitamins B1 and B6 to the PTZ-treated animals on the levels of amino acids and urea in the cerebral cortex of the male and female rats next day after the PTZ-induced seizure. The metabolites are indicated on the Y axes, their content is expressed in micromoles per g of tissue.

Effects of the administration of vitamins B1 and B6 to the animals not exposed to PTZ.

In view of the revealed enhancement of the delayed PTZ effects by the vitamins shown above, the male and female rats not exposed to PTZ, i.e. the control groups, have been treated with the vitamins according to the same scheme as the PTZ-treated animals (Supplementary Figure S1). As

seen from Figure 7, the female rats are not affected by the vitamins administration, but the male rats demonstrate an increase in anxiety ($p = 0.05$) (Fig. 7). Besides, the administration of vitamins induces gender differences in anxiety and exploratory activity (Fig. 7). These effects of the vitamins administration are similar to the delayed effects of PTZ administration (Fig. 1 B).

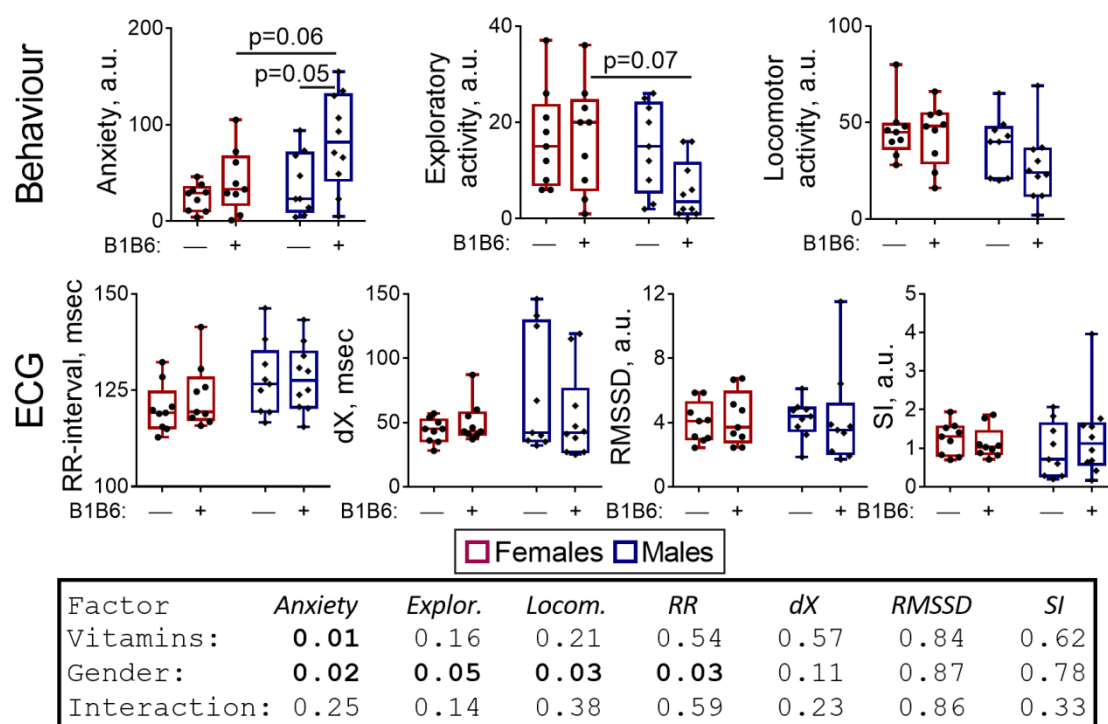
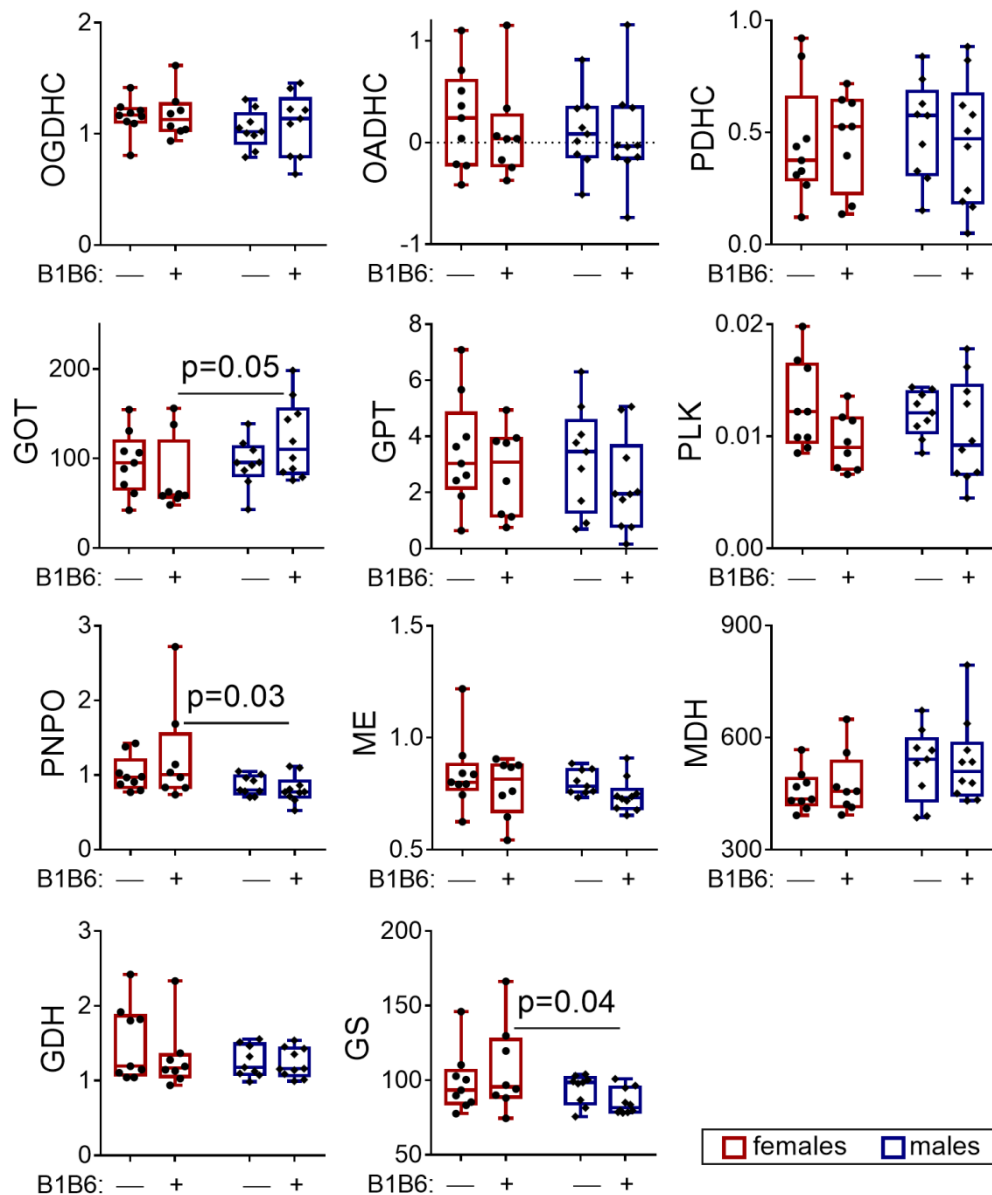


Figure 7. Physiological effects of administration of vitamins B1 and B6 to the control rats. The studied parameters are indicated on Y axes and described in details in Figure 1 and “Materials and methods”.

The gender-dependent effects of administration of the vitamins on physiological parameters of the control rats (Fig. 7) reciprocate those on the enzymatic activities, revealing sexual dimorphism in the levels of GOT, PNPO and GS after the vitamins administration (Fig. 8). A significant ($p = 0.05$) effect of the vitamins on the PLK activity regardless of gender is also revealed by two-way ANOVA (Fig. 8). Comparison of effects of the vitamins on the amino acid profiles in the control rats (Fig. 9) and those treated with PTZ (Fig. 6) indicates that the vitamins similarly affect the gender relationships in the levels of GABA and alanine in both the PTZ-treated (Fig. 6) and control (Fig. 9) animals. However, in the PTZ-treated animals this effect of vitamins antagonizes the opposite effect induced by PTZ (Fig. 3). On the other hand, some of the vitamins effects in the control rats, such as a decrease in phenylalanine in the female rats and abrogation of the gender difference in aspartate (Fig. 9), are similar to those of PTZ (Fig. 3).



Factor	<i>OGDHC</i>	<i>OADHC</i>	<i>PDHC</i>	<i>GOT</i>	<i>GPT</i>	<i>PLK</i>
Vitamins:	0.68	0.60	0.80	0.67	0.18	0.05
Gender:	0.17	0.57	0.82	0.11	0.55	0.93
Interaction:	0.85	0.80	0.66	0.10	0.84	0.48
Factor	<i>PNPO</i>	<i>ME</i>	<i>MDH</i>	<i>GDH</i>	<i>GS</i>	
Vitamins:	0.31	0.12	0.67	0.36	0.99	
Gender:	0.85	0.32	0.04	0.20	0.04	
Interaction:	0.22	0.93	0.83	0.47	0.18	

Figure 8. Effects of administration of vitamins of B1 and B6 to the control rats on the enzymatic activities in the rat brains. The activities are expressed in micromole/min per g of tissue, with the exception for the PNPO activity, expressed in nanomole/min per g of tissue.

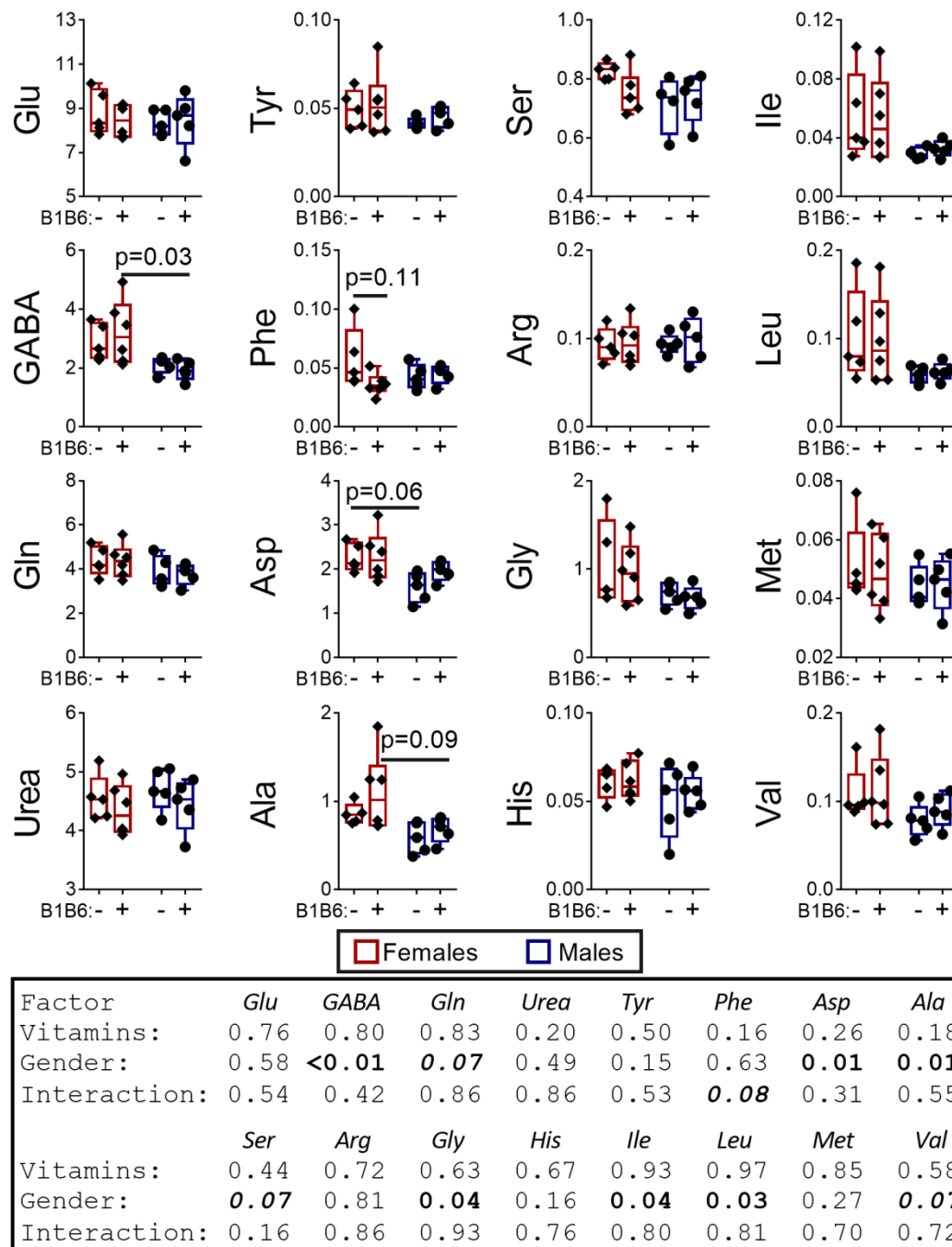


Figure 9. Effects of administration of vitamins of B1 and B6 to the control rats on the levels of amino acids and urea in the rat brain. The content of free amino acids and urea is shown in micromoles per g of tissue.

Accordingly, these effects of PTZ are not normalized by the vitamins supplementation to the PTZ-treated animals (Fig. 6), reciprocating certain similarity in the physiological effects of vitamins (Fig. 7) or PTZ (Fig. 1 B). However, most of the gender effects induced by the combined action of the vitamins and PTZ on the brain amino acid levels (Fig. 6), are not observed upon the action

of each of the factors (Fig. 3, 9), thus arising due to synergistic action of vitamins and PTZ upon their combined administration.

Interval between administration of vitamins B1/B6 and PTZ influences the PTZ-induced seizure and its consequences in females.

In view of a higher sensitivity of female rats to both PTZ and antiepileptic action of the vitamins (Fig. 1, 4), the time-dependence of the administration of vitamins B1 and B6 before PTZ has been studied in female rats, comparing the “long-term” (24 h) and “short-term” (2 and 0.5 h) procedure of the vitamins administration (Supplementary Figure S1). In contrast to the antiepileptic action of the vitamins administered 24 h before PTZ, the administration of vitamins 0.5 or 2 h before PTZ has a pro-epileptic effect. The animal group with vitamins administered 0.5 h before PTZ has been the only one with increased rate of deadly seizure, in contrast to 100% survival in other groups (see “Animal survival” in “Materials and Methods”). Stronger seizures in these rats are confirmed by a lower dose of PTZ causing seizures and an earlier onset of twitching and tonic seizures, compared to the rats, receiving vitamins 24 h before PTZ (Fig. 10 A). The time dependence of the action of vitamins in females confirms the difference in the effects of the short-term and long-term administration of vitamins, pointing to the opposite physiological effects of the administration 2 and 24 h before PTZ (Fig. 10 A). Thus, the vitamins administered 0.5-2 h before PTZ, sensitize female rats to the action of PTZ, while the vitamins administered 24 h before PTZ has an anti-epileptic effect.

Delayed consequences of an epileptic seizure in females further confirm the opposite effects of the vitamins administration 2 and 24 h before PTZ. This is observed as a significant drop in exploratory activity and an increase in the RR-interval of ECG in the rats with the short-term vitamins administration, compared to the rats which received vitamins 24 h before or do not receive vitamins at all (Fig. 10 B). The low level of exploratory activity in the groups of rats receiving the vitamins administration 0.5 or 2 h before PTZ (Fig. 10 B) is consistent with the parameters of seizures, indicating that administration of vitamins at these time points sensitizes the female rats to PTZ. Overall, physiological indicators of the delayed effects of a seizure are in good agreement with the pathophysiological parameters of the seizure itself, testifying to the facilitation of a seizure after the short-term administration of vitamins prior to PTZ, and a protective effect of the vitamins administered 24 h before a seizure (Figure 10 A).

Remarkably, the decrease in the PLK activity in the brain of female rats after administration of the vitamins 24 h before PTZ (Figs. 7, 10 B), is absent at short intervals between administration of the vitamins and PTZ (Fig. 10 B). Moreover, administration of vitamins 0.5 h before PTZ causes an increase ($p = 0.05$) in the PLK activity, compared to the long-term administration of the vitamins.

Thus, the activation of PLK upon the short-term administration of vitamins corresponds to the physiological sensitization of rats to PTZ, whereas a decrease in the PLK activity in response to the long-term administration of the vitamins is associated with the protective effect of vitamins against the PTZ-induced seizure.

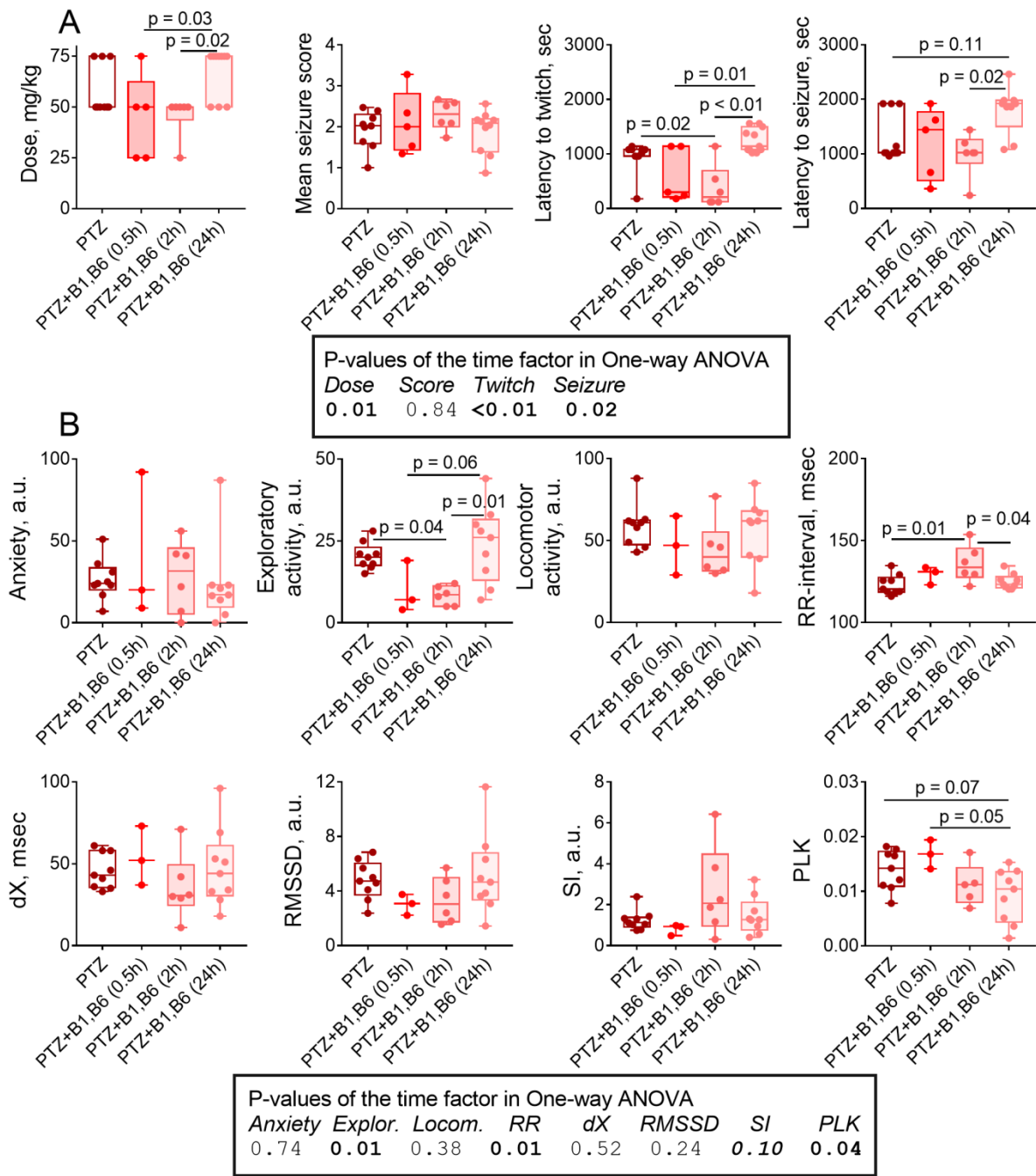


Figure 10. Dependence of pathophysiological parameters of the PTZ-induced seizure (A) and its delayed physiological and biochemical effects (B) in female rats on the interval between the administration of vitamins B1/B6 and PTZ. The assayed parameters are indicated on the Y axes and described in “Materials and methods”. The activity of the female brain PLK (micromole/min per g of tissue) is used as an indicator of biochemical changes.

Discussion.

Gender differences in epilepsy and underlying molecular mechanisms remain a well-known challenge for therapeutic approaches [17,25]. Accumulating data point to significant interactions between the hormonal status and other factors in epilepsy [17], which should be taken into account to improve therapy of epilepsy in a gender-dependent manner.

While most of the clinical and experimental studies in epilepsy focus on seizures themselves, the delayed consequences of seizures are not at all less important, as accumulation of even minor changes in the brain homeostasis may lead to a vicious cycle deteriorating the patients state along time. Employing the PTZ-induced model of epilepsy we therefore analyze not only the parameters of seizures themselves, but also their physiological consequences and biochemical basis the day after. By comparison of a seizure in the male and female rats we show that the different reaction of the genders to the epileptogenic and protective factors is not always obvious as significant effects in each of the group, but may be manifested as an induction of gender differences, or contrary, as disappearance of such differences. Both events obviously result from minor gender-specific changes in opposite directions, which do not reach statistical significance in each gender. The comparison of the males and females is therefore more powerful to detect the gender-specific effects. Using this comparative analysis, we have characterized induction or abrogation by PTZ and/or vitamins of gender differences in physiological and/or biochemical parameters, absent or pre-existing, correspondingly, in the non-affected state. The data obtained in this work show that such cumulative effects are much more pronounced, than specific actions of PTZ and/or vitamins in the female or male rats. For instance, in our study of the action of vitamins on the PTZ-treated animals (Fig. 6), eight statistically significant ($p \leq 0.05$) gender differences in the amino acid profiles are affected by the vitamins (of those six arise and two disappear). However, the statistically significant effects of the vitamins on each gender include only two changes in the male rats and none in the female rats.

It is worth noting that, based on both the enzymatic activities (Fig. 2) and amino acid profiles (Fig. 3), there are more PTZ-induced changes in the brain of the males, compared to the females, which coincides with a lower PTZ sensitivity of the male than female rats (Fig. 1). Thus, more metabolic changes in the brain correspond to less expressed physiological effects. This feature has been observed by us in another model [23], with both the previous and current data supporting a notion that the biochemical changes in the brain enzymes and amino acids, observed in these models, manifest adaptation rather than damage.

All together our experiments reveal multiple gender-specific responses in epilepsy and therapeutic approaches to its treatment. Such responses are revealed in the epileptogenic action of PTZ, delayed consequences of a seizure and action of vitamins B1 and B6. Changes in the brain amino

acids provide a very sensitive indicators of the cumulative physiological responses, while the *in vitro* assays of the brain enzymes reveal the most affected parts of metabolic networks. Compared to the control rats, PTZ action increases the brain PDHC in both genders, decreasing MDH only in the male rats. Combined with a gender-specific expression of GDH, these subtle differences in the functional expression of the three out of eleven tested brain enzymes of central metabolism (Fig. 2) are accompanied by multiple changes in the brain amino acids, including glutamate and GABA which are of utmost significance in epilepsy (Fig. 3).

Thus, delayed consequences of a seizure involve changed activities of central metabolic enzymes and associated amino acid metabolism. Dependence of such metabolism on vitamins may result in so called metabolic epilepsies often positively responding to administration of vitamins [26]. While deficiencies in vitamins B1 and B6 due to malnutrition have not been considered an issue in the developed countries since a long time, these states currently become more and more often as a result of intensified medical treatments, such as administration of diuretics, metformin, antibiotics, chemotherapy, bariatric surgery [13,16,24,27]. Regarding vitamins B1 and B6, seizures are known to arise due to mutations in their transport, metabolism and physiological function [28-31]. In the diagnosed cases of neonatal epilepsies originating from the pathogenic mutations, urgent administration of the required vitamin is essential to prevent not only seizures, but also further mental retardation [30-32]. It should be noted, however, that less pathogenic mutations in the proteins of vitamin metabolism may be compensated under normal physiological conditions, yet their pathogenicity may increase under stress and/or medical interventions. This feature may underlie interindividual variations in developing the vitamins deficiencies promoted by pathophysiological conditions. In such cases, pharmacological doses/forms of vitamins may be a valuable therapeutic option. However, unlike vitamin B1, whose high pharmacological doses do not usually have damaging effects [13], high doses of vitamin B6 are neurotoxic [29]. Therefore, in view of our finding of the mutual regulation of metabolism of vitamins B6 and B1 [11], the antiepileptic action of the combined administration of both vitamins is studied in this work. Nevertheless, a potentiating effect of the combined administration of vitamins B1 and B6 on seizures is observed in our study upon the vitamins administration shortly before PTZ in female rats (Fig. 10, Table 2). Besides, the vitamins increase anxiety in the control male rats (Fig. 7). These negative effects of the combined administration of B1 and B6 may be ascribed to the action of vitamin B6. Such a view is supported by the transformation of the pro-epileptic effect of the vitamins administration into a protective one along with downregulation of the B6-dependent PLK in the brain of female rats (Fig. 10), and increased gender differences in the B6-dependent enzymes GOT and PNPO after the vitamins administration to the control animals (Fig. 8). However, the

vitamins-induced reversal (Fig 6) of the PTZ effects on the gender differences in glutamate, GABA, glutamine and alanine (Fig. 3) points to their therapeutic action in combating the delayed consequences of a seizure, warranting further studies.

Materials and Methods.

Reagents from the following manufacturers were used: thiamine – «Serva», Germany; pyridoxal – «PanReac AppliChem», Spain; glycerol – «Biomedicals, LLC», USA; pentylenetetrazole (PTZ), protease inhibitors, buffers and other reagents for the analysis of enzyme activities – «Sigma-Aldrich», USA. Pyridoxine-5'-phosphate was synthesized from pyridoxal-5'-phosphate («Sigma-Aldrich», USA) as described previously [33]. The solutions were prepared in Milli-Q deionized water, the salts used were of the highest purity available.

Animals

The studies were carried out in Wistar rats obtained from the Russian Federation State Research Center Institute of Biomedical Problems RAS (IBMP) - both males and females, weighing 250-350 g. The animals were kept under standard conditions in cages with free access to water and food and a light/dark cycle of 12/12 hours (the light turned on at 9 a.m.). The adaptation period to the husbandry conditions was two weeks. The age of the animals at the time of the study was 2.5-3.0 months. Animals were distributed randomly between experimental groups. Animal experiments were approved by Bioethics Committee of Lomonosov Moscow State University (protocol number 69-o from 09.06.2016).

Pentylenetetrazole model of epileptic seizures

Convulsions were induced by intraperitoneal administration of PTZ in saline at 25 mg/kg dose. After PTZ administration, the severity of seizures was visually assessed according to the Racine scale [34] (Table 1) for 15 minutes in individual cages (OpenScience, Russia). If within 15 minutes, the stages 4-5 on the Racine scale (tonic or tonic-clonic seizures, Table 1), did not develop, the PTZ was re-injected at the same dose of 25 mg/kg. The procedure was repeated no more than three times, and the total PTZ dose thus did not exceed 75 mg/kg. The total time of the seizure observation was 45 minutes (Supplementary Figure S1).

Table 1. Racine scale for visual assessment of the severity of epileptic seizures in rats.

Score	Behavioral manifestations of seizures
0	Normal behavior, no abnormality
1	immobilization, lying on belly
2	head nodding, facial, forelimb or hindlimb myoclonus
3	myoclonic twitches, continuous whole-body myoclonus, tail held up stiffly
4	rearing, tonic seizure, falling down on a side
5	tonic-clonic seizure, falling down on back, wild rushing and jumping

The mean seizure score was calculated as the average score of the severity of an epileptic seizure over the entire observation period from the moment of administration of the first PTZ dose.

Vitamin administration

Vitamins B1 (thiamine, 100 mg per kg body weight) and B6 (pyridoxal, 100 mg per kg body weight) were administered intraperitoneally 24 hours, 2 hours or 30 minutes before the first PTZ administration and after completion of a 45 minutes follow-up of the PTZ-induced seizures (Supplementary Figure S1). This vitamin regimen took into account the results of previous studies on the potential protective effect of high doses of vitamins both before [35] and after [22] the exposure to stress, when increased availability of vitamins may provide, respectively, better stabilization and normalization of the metabolic state [36]. Control animals received the injections with equivalent volumes of physiological solution (0.9% NaCl). Since the epileptic seizure occurs before the second injection of vitamins, only the first injection of vitamins affects the convulsions themselves. Both the first and the second doses of vitamins affect the delayed consequences of a seizure.

In the comparison of the male and female rats, the first administration of the vitamins was performed 24 h before PTZ. The study of the time-dependence of the vitamins administration was performed on the female rats only. A significant part (about 70%) of both vitamins is known to be excreted from the body 24 h after the injection [37,38]. However, the injections significantly increase the internal pool of vitamins [39] that can be stored in the liver, where concentrations of thiamine diphosphate and B6 vitamers are higher than in other tissues [14,40].

According to the formula recommended by the US Food and Drug Administration (<http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm078932.pdf>), the dose of vitamins B1 and B6 used in this study on rats (100 mg/kg each of the vitamins) corresponds to a dose of 16 mg/kg, or 1 g for an average weight of 60 kg, in humans. No toxic or side effects in humans are known for vitamin B1 at a daily dose of 0.5 g for a month [41]; clinically used doses of vitamin B1 may be up to 0.5 g injections three times a day [42]. However, high doses of vitamin B6 are neurotoxic [38,43]. For instance, 2-6 g of vitamin B6 per day, resulted in acute sensory neuropathies [38,44]. At the same time, such effects were not reported in patients with various B6-dependent syndromes who received 0.5-1.5 g of vitamin B6 per day [38]. Thus, the dose of vitamins B1 and B6 used in our study are within interval of the doses clinically used in megavitamin therapy. In addition, taking into account the inhibition by vitamin B1 of pyridoxal kinase [11], which produces the coenzyme form of vitamin B6, the

coadministration of vitamins used in this study may help to reduce the B6 neurotoxicity, potentially linked to production of the coenzyme pyridoxal-5'-phosphate by PLK.

Animal survival

The data on the total number of animals in our experiments and their survival is summarized in Table 2.

Table 2. Initial number of animals and their survival in each group.

Animal gender	Group	n	Survival, %
Females	PTZ	9	100
	PTZ +B1,B6 (24 h)	9	100
	PTZ +B1,B6 (2 h)	6	100
	PTZ +B1,B6 (30 min)	5	60
	B1,B6 (24 h)	8	100
	Control	9	100
Males	PTZ	10	100
	PTZ +B1,B6 (24 h)	12	100
	B1,B6 (24 h)	10	100
	Control	9	100

Assessment of physiological parameters

To assess the spontaneous behavior of animals in an unfamiliar environment, we used the «Open Field» test («OpenScience», Moscow, Russia) [45]. The animals were tested in complete silence under the light of a 15 W red lamp for three minutes, assessing the following indicators: horizontal locomotor activity (the number of segments passed); rearing or vertical locomotor activity (the number of stands on the hind limbs); steps out of the walls (the amount of movements from the walls intersecting the outer circle) and entries to the center of the field (the number of movements to the center of the arena intersecting the outer and inner circles); total freezing time; the number of acts and the total time of grooming; the number of defecation acts. The parameters were further combined into the three categories, presented in the graphs, as further described. Locomotor activity was assessed as the number of segments passed; exploratory activity summarized the numbers of rearings, steps out of the walls and entries to the center; anxiety summarized grooming and defecation acts with the grooming and freezing times. The employed combination of parameters to characterize anxiety was based on the studies showing their unidirectional increase, accompanied by reductions in the locomotor and/or exploratory activities [46-49]. All cumulative indicators were assigned arbitrary units, such as, e.g., 1 s of freezing = 1 a.u.; 1 act of defecation = 1 a.u., etc.

ECG was recorded using cutaneous electrodes (disposable silver chloride electrodes for recording ECG in newborns), attached to the right and left on the ventrolateral surface of the animal's chest. Prior to this, the skin in these areas was degreased with alcohol and the electrodes were glued, fastened to the terminals, mounted on a harness of the original design, which was put

on the animal. The connector was linked to a biopotential amplifier having a frequency range of 10 Hz to 20 kHz. The signal from the electrodes was transmitted to an E14-440 analog-to-digital converter (L-Card, Moscow, Russia) connected to a computer. The analog signal was recorded from free-roaming rats using the Powergraph software (Russia, DISoft LLC), digitized at a frequency of 1 kHz and processed using Spike-C3, Average and Ints software («OpenScience», Moscow, Russia).

The balance of autonomous regulation of heart rate, representing the relative contribution of the sympathetic and parasympathetic components to the activity of the nervous system, was assessed in accordance with Baevsky et al. [50]. The following parameters of the heart rate variability were calculated: average R-R interval in a sample, ms; dX - variance of RR-intervals in a sample, ms; parasympathetic, or relaxation, index of the state of the nervous system – RMSSD; sympathetic, or stress, index of the state of the nervous system – SI [50,51].

Preparation of homogenates of the rat cerebral cortex

Homogenization of the tissue and sonication of homogenates was carried out according to the previously published protocol [52].

Preparation of tissue extracts and quantifications of metabolites

Methanol-acetate extraction of the rat cerebral cortex and quantification of its amino acids and urea were done as described before [23,53]. Briefly, frozen cerebral cortices were homogenized in ice-cold methanol, then acetic acid solution was added and the proteins were precipitated. The sodium-acetate buffer system with a Hitachi L-8800 amino acid analyzer was used according to the manufacturer's User Manual, as described before [23,53].

Measurement of enzymatic activities

The activities of thiamine-diphosphate-dependent enzyme complexes of 2-oxoglutarate dehydrogenase and 2-oxoadipate dehydrogenase, as well as the activities of GDH, MDH, GS and ME were measured as previously described [21]. The activity of the pyruvate dehydrogenase complex was determined according to a published protocol for the enzyme assay in tissue homogenates [54]. The activities of aspartate aminotransferase and alanine aminotransferase were measured according to the method of Reitman and Frankel [55]. All of the above activities were assayed in 0.2 ml of the reaction medium spectrophotometrically in transparent 96-well microplates, using a Sunrise plate reader (Tecan, Austria).

The activity of PLK was measured using a published assay system with several modifications [56]. 2-8 μ l of a rat cerebral cortex homogenate with a protein concentration of about 50 mg/ml were added to 0.2 ml of the reaction mixture comprising 50 mM KH_2PO_4 , pH 6.0; 0.1 mM ZnSO_4 ; 0.2 mM pyridoxal; 2 mM Na_2ATP . The mixture was incubated at 37 °C for 90 min, followed by the cooling of the plate on ice for 5 min and immediate addition of a cold solution of

6 mM hydroxylamine to a final concentration of 1 mM. A control medium without ATP was used as a blank. The fluorescence (ex365nm | em450nm) was measured 150 s after the hydroxylamine addition during 5 min. The calculation of the rate of the PLK reaction was carried out as described [56].

Our assay of PNPO was based on the enzymatic production of hydrogen peroxide, which may be detected in complex biological mixtures by Amplex UltraRed (Thermo Fisher Scientific, USA) [57]. The reaction was carried out at 25 °C in 0.2 ml of the reaction mixture comprising 50 mM HEPES, pH 7.4; 50 mM KCl; 10 mM NaCl; 10 µM pyridoxine 5'-phosphate; 5 U/ml horseradish peroxidase; 10 µM Amplex UltraRed (diluted from 10 mM stock solution in DMSO). To measure the reaction blank, the reaction medium omitting pyridoxine 5'-phosphate was used. The reactions were started with 3-15 µl of the rat cerebral cortex homogenate. The fluorescence (ex560nm | em590nm) was measured during the 10 minutes of the reaction.

The fluorescent assays of PLK and PNPO were done in black microplates using a CLARIOStar plate reader (BMG LABTECH, Germany).

Statistical analysis and data presentation

Data were analysed using the GraphPad Prism 7.0 software (GraphPad Software Inc., USA). Individual values of the parameters for each animal, their median, quartiles and values of the minimum and maximum are shown on the graphs. Statistical significance of differences between the two groups was assessed using the Mann-Whitney U-test. Statistical significance of differences upon comparison of more than two experimental groups was assessed using one-way or two-way analysis of variance (ANOVA) with Tukey's post hoc test. The values of $p \leq 0.1$ are shown in the graphs. The tables below the figures show the ANOVA results for the significance of factors and their interaction; significant ($p \leq 0.05$) values are in bold, trends ($0.05 < p \leq 0.1$) are in bold italics.

Supplementary Materials

Supplementary Figure S1. Flowchart of physiological experiments in intact rats (A), rats receiving vitamins B1 and B6 (B), rats receiving PTZ without the vitamins (C), rats receiving PTZ with the vitamins (D). The female and male rats received 100 mg/kg of each of thiamine and pyridoxal intraperitoneally 24 h before the first administration of PTZ. The same experimental design was employed in the study of the time dependence of the vitamins effects, which was done on the female rats, where the time of the first administration of the vitamins was 24, 2 or 0.5 h before PTZ (D). PTZ was administered at a dose of 25 mg/kg, followed by estimation of the severity of a seizure during 15 minutes (Table 1). In case of a weak seizure (0-3 score points, Table 1), PTZ administration and seizure assessment were repeated up to three times. In total, the PTZ dose didn't exceed 75 mg/kg. After 45 minutes of the seizure observation, vitamins B1 and B6 (100 mg/kg each) were injected once again. After 24 hours, the physiological parameters were assessed using an "open field", the ECG was recorded, and the rats were decapitated. The cerebral cortex was quickly removed and frozen in liquid nitrogen (60-90 sec after decapitation). In the control groups the injections of vitamins and/or PTZ were substituted by injections of equivalent volumes of physiological solution (0.9% NaCl).

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Author Contributions

Conceptualization, V.B.; All the co-authors contributed to Methodology, Validation, Formal Analysis, Investigation, Resources, Data Curation; Writing – Original Draft Preparation, V.A. and V.B.; Writing – Review & Editing, V.B. with all co-authors; Visualization, V.A. and A.G.; Supervision, V.B.; Project Administration, V.B.; Funding Acquisition, V.B.

Institutional Review Board Statement: All animal experiments were performed according to the guidelines of the Declaration of Helsinki and were approved by Bioethics Committee of Lomonosov Moscow State University (protocol number 69-o from 09.06.2016).

Data Availability Statement: The data presented in this study are available in this article (summarized in figures and Tables, including Supplementary data). The raw data are available on request from the corresponding author.

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Abbreviations:

PTZ – pentylenetetrazole
 OGDHC – 2-oxoglutarate dehydrogenase complex
 OADHC – 2-oxoadipate dehydrogenase complex
 PDHC – pyruvate dehydrogenase complex
 GDH – glutamate dehydrogenase
 MDH – malate dehydrogenase
 PLK – pyridoxal kinase
 PNPO – pyridoxal 5'-phosphate oxidase;
 GS – glutamine synthetase
 ME – malic enzyme
 GOT – glutamate-oxaloacetate transaminase
 GPT – glutamate-pyruvate transaminase
 GABA – gamma-aminobutyric acid

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